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Freund's Adjuvants: Relationship of Arthritogenicity and Adjuvanticity in Rats to Vehicle Composition

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Summary. Over a hundred compounds and natural materials were examined for their ability to induce arthritis in rats when mixed with heat-killed delipidated *Mycobacteria tuberculosis*. Many of these materials were also assessed for (CMI) adjuvant activity by their ability to induce allergic encephalomyelitis (EAE) in rats when mixed with guinea-pig spinal cord, both with and without added *M. tuberculosis*.

Cyclization and/or the presence of oxygen atoms, or double bonds reduced (or abolished) the arthritogenic potential and adjuvanticity of alkanes $> C_{10}$. Esters/triglycerides of fatty acids $> C_{12}$, retinol acetate (not palmitate) and vitamins E and K showed co-arthritogenic and adjuvant activity. Other active lipids included squalene and cholesterol oleate, which are both present in human sebum. Sebaceous lipids may therefore perhaps function as natural adjuvants if resorbed during abrasion and infection.

Squalane (perhydrosqualene), pristane and hexadecane were excellent substitutes for mineral oil in preparing arthritogenic adjuvants from various mycobacteria, *C. rubrum* and *N. asteroides*. These oily compounds were also very effective adjuvants *per se*, in the absence of bacterial material or emulsifier, for inducing EAE in Lewis rats.

The use of mycobacteria with mineral oil and an emulsifying agent to constitute immunological adjuvants (Asherson and Allwood, 1969; Freund, 1947, 1956; White, 1967, 1972) has gained widespread acceptance for enhancing both the cell-mediated and humoral responses to a variety of antigens. It is convenient, though perhaps not always accurate, to distinguish adjuvants composed of dried bacteria and an oily vehicle (both with or without an emulsifier) by designating them Freund's (-type) adjuvants; in contradistinction to other types of immunological adjuvants, e.g. mineral earths, endotoxins, oligonucleotides, methylated albumin, calcium alginate, etc.

Such Freund's adjuvants, prepared from certain heat-killed species of *Mycobacteria*, *Corynebacteria* and *Nocardia* by dispersion in mineral oil alone (i.e. no emulsifier), will induce a chronic polyarthritis 10 days or more after being injected in the footpad or tail or

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ears of sensitive rat strains (Pearson, 1972). This so-called 'adjuvant disease' provides a useful model for evaluating potential anti-arthritic agents, but is complicated by the involvement of many organs other than the joints, notably the liver, bone marrow, spleen, etc. This complex disease is only induced by a combination of an arthritogenic micro-organism and a suitable oily vehicle; administering the same organism for example in a saline suspension fails to induce the arthritis.

While a number of bacterial species and subfractions therefrom have been screened for their ability to induce adjuvant arthritis when co-administered with mineral oils, comparatively little interest seems to have been evinced concerning the molecular determinants of co-arthritogenicity associated with the oily vehicle. Because of the complexity, non-sterility and variable composition of mineral oil from different sources, we have sought chemically defined alternative media for injecting arthritogenic bacteria. A measure of the intrinsic 'adjuvanticity' of the oily vehicle was obtained by determining its activity as a 'co-encephalitogen', i.e. ability to induce allergic encephalomyelitis when administered to Lewis rats, together with guinea-pig spinal cord, without any adjuvant bacteria. To minimize the number of variable factors, all these studies were conducted without using any emulsifying agents.

Preliminary reports of these studies have appeared elsewhere (Whitehouse, Whitehouse, Vande Sande and Pearson, 1971; Whitehouse, Orr, Beck and Pearson, 1973).

MATERIALS AND METHODS

Two strains of outbred male Wistar rats (CFN from Carworth Farms, New City, New York and HLW from Hilltop Labs, Chatsworth, California) were mainly used to study development of arthritis. Two strains of inbred male rats (Lewis from Microbiological Associates, Walkersville, Maryland and Fischer-344 from Hilltop Labs) were used for studies of allergic encephalomyelitis (EAE). Groups of four or five animals were bedded on sawdust and allowed free access to food.

A heat-killed mixture of three strains (C, DT, PN) of *Mycobacteria tuberculosis* (human) was obtained from the Central Veterinary Laboratory, Weybridge, England through the courtesy of Dr B. B. Newbould (ICI Pharmaceuticals Division, Macclesfield, England). These dried cells were delipidated by three successive extractions with ethanol–ether (1:1 v/v). Heat-denatured *Nocardia asteroides* and *Corynebacteria rubrum* were kindly supplied by Dr R. Perrin (Calbiochem, San Diego, California). Purified preparations of cell walls from *Mycobacteria bovis* (BCG) were generously donated by Dr G. B. Mackaness, Trudeau Institute, Saranac Lake, New York.

Vegetable oils were purchased in a local market or obtained from Nutritional Biochemical Company, Cleveland, Ohio. Mineral oils were refined commercial products (Squibb, Witco) for medicinal use. Paraffins, olefins and other chemicals were purchased from the Aldrich Chemical Company (Milwaukee, Wisconsin), Sigma Chemical Company (St Louis, Missouri), or Eastman Kodak Company (Rochester, New York). Solid materials, e.g. waxes, were liquefied by admixture with, or solution in, bland solvents (hexane, benzene) if they failed to melt on warming to 33°. Unsaturated oils were brominated in methanol at 4° and the lower layer collected after 3 hours and freed of methanol by aspiration with dry air. Squalene was purified via the thiourea adduct (Knight, Witnauer, Coleman, Noble and Swern, 1952) and used immediately after decomposing this adduct with water. The squalene was extracted into petroleum ether (b.p. 60/80°)

and recovered after evaporating the petroleum ether with a stream of nitrogen and warming. Anti-oxidants (hydroquinone, butylated anisole) were added to minimize re-oxidation at this stage.

Preparation of 'adjuvants'

Dried bacteria (listed above) or BCG cell walls were crushed in a mortar and finely ground with slow continuous additions of the oily vehicle under study; the whole operation taking at least 10 minutes. Sufficient oil or solubilized wax was added to give final concentrations of bacteria = 10 mg/ml, or cell walls = 4 mg/ml, in the resulting dispersion. These dispersions (anhydrous adjuvants) were kept at 4° and were stable, as far as their biological properties were concerned, on prolonged storage at 4°. Sedimented material readily redispersed on warming and shaking. Adjuvant preparations failing to meet this criterion were discarded. No preservatives or emulsifying agents were added to any of these dispersions.

Evaluation of adjuvants

For arthritogenicity. Each preparation was assayed by injecting 50 μl/animal into the tail of one group of rats and into the right rear paw of another group of 180–250 g Wistar rats (on day 0). Occasionally adjuvants were also inoculated directly into the inguinal nodes as described below, approximately 5 μl being injected into each node (i.e. 10 μl/animal). Animals were weighed at intervals (day 0, 3, 7 and 14) and paw thickness evaluated at these times with a micrometer screw gauge. Selected groups of animals received sodium hexobarbital (100–150 mg/kg) i.p. at occasional intervals and their sleeping times in response to this hypnotic were recorded to the nearest 5 minutes. Other parameters of adjuvant disease which were evaluated on days 3 or 14 were serum albumin, fibrinogen and plasma inflammation units (Glenn and Kooyer, 1966). Splenomegaly and size of lymph nodes were determined on killing the animals after day 16. The capacity of liver homogenates to metabolize aminopyrine and cyclophosphamide *in vitro* was determined at day 2 or 3 or day 14 onwards as described (Beck and Whitehouse, 1973), using selected groups of animals.

For adjuvanticity (in promoting the development of EAE). Each bacterial adjuvant was diluted with nine volumes of the homologous oily vehicle under study to give a 'thin' adjuvant which was usually too dilute to cause consistent adjuvant disease, i.e. arthritis. Equal volumes of (i) this thin adjuvant or (ii) the oily vehicle alone, were mixed at room temperature with an equal volume of freshly thawed guinea-pig spinal cord in a 6-ml syringe and the whole admixture was emulsified first by agitation of the contents of the syringe with a Vortex mixer (Scientific Products, Evanston, Illinois) and then by forcing it repeatedly through a 30-gauge stainless steel double-headed needle inserted between two 1-ml syringes. This process was continued for at least 5 minutes and until the mixture acquired the appearance and consistency of a hand lotion and was uniformly white. This formulation was then tested by adding two drops to a dish of water; if the drops did not disintegrate (with spreading of the oil only), the emulsion was considered satisfactory for further study. No exogenous emulsifying agents were used. This encephalitogenic emulsion was always used immediately after preparation and injected via a 30-gauge, 1-inch needle directly into both pre-exposed inguinal nodes (Newbould, 1965) of 170–250 g Lewis or Fischer rats anaesthetized with ether. The total amount of emulsion injected per animal was usually 10–20 μl; the injection was continued until the emulsion could be seen to be

leaving the node and moving up the draining lymphatic vessel. The incision was closed with wound clips and liberally douched with 1 per cent aqueous picric acid. Animals were routinely weighed on day 0, day 7 and daily thereafter. Signs of disease were evaluated by noting the onset of paralysis (loss of tail flexion succeeded by hind limb paralysis), weight loss and ultimately, time of death or rate of recovery. A paralytic score was computed for each animal at any one day being compiled by assigning 1 point for complete tail 'flop' (0·5 if only partial), 1 point for hind limb paralysis, 1 point for each 20 g loss in weight compared to the highest weight recorded over days 5 to 9, 1 point for death; the maximum score being 6 per animal.

For chemical composition. The original oily vehicles and their derived adjuvants were analysed for rancidity (i.e. epoxidation) by taking 0·1 ml for colorimetric analysis for epoxides using 2-thiobarbituric acid (Wilbur, Berheim and Shapiro, 1949; Sharma and Krisha Murti, 1963). The degree of unsaturation (iodine number) was determined with bromine (Benham and Klee, 1950), using fumaric acid as a standard.

RESULTS

PREFATORY NOTE

These experiments were conducted over a 3-year period. Every attempt was made to conform to a consistent protocol of animal care (feeding, cleaning) and handling (inoculation, weighing, etc.) to control as many variables as possible. The responsiveness of different batches of animals was checked by routinely inoculating a group of four or more animals with a standard arthritogenic adjuvant prepared at regular intervals from a stock of ethanol-extracted *M. tuberculosis* and a sterile mineral oil ('Protol', Witco) supplied by

TABLE 1

RESPONSE OF FOUR RAT STRAINS TO ONE HIGHLY ARTHRITOGENIC ADJUVANT

Strain	Weight gain (g) days 0–14 (±SEM)		Percentage inhibition of weight gain	Arthritis† day 14
	No adjuvant	With adjuvant		
Fischer 344	44±5	10±17	77	++
CFN Wistar	62±11	10±8	84	+++
HLW Wistar	105±15	30±7	72	+++
Lewis	38±7	−8*±14	100+	++++

The adjuvant was *M. tuberculosis* in 50 μl of sterile mineral oil, administered on day zero.

The data was collected from groups of eight rats initially weighing 180–210 g.

* Net weight loss.

† Arthritis scores in this and subsequent tables record the severity of external signs as follows: 0 = none; ± = either erratic incidence of a definite arthritis, or a more uniform but still questionable response to adjuvant; + = either irregular incidence of ++ disease, or a consistent minimal incidence of lesions on non-injected feet, but enduring and prominent swelling of adjuvant-inoculated foot; ++ = consistent mild disease affecting non-injected feet (but causing only modest swelling thereof) in 75 per cent of the inoculated animals; +++ = normal consistent severe disease, affecting all limbs (day 14) and often the tail (day 17) with frequent ear lesions; ++++ = gross swelling of all extremities, inflamed scrotum, prominent ear lesions, frequent balinitis, but never lethal.

the UCLA Hospital Pharmacy. Each of the four strains of rats used for these experiments responded to this reference adjuvant with a polyarthritis (paw swelling) of greater than 80 per cent incidence in both rear paws (after 2 weeks) and greater than 60 per cent

TABLE 2

ARTHRITOGENIC ACTIVITY OF NORMAL HYDROCARBONS AND THEIR DERIVATIVES, IN CONJUNCTION WITH *M. tuberculosis*

Compound	Severity of arthritis†	Sleep time* (days 13–17)	DMA‡	Percentage average weight gain§	Comment (alternative name)
(A) Hexane	0	N	n	100	? Positive skin test (day 5) to PPD
Octane	0		d	97	
Nonane	0	N	n	72	
Decane	+	E	dd	75	
1-Decene	±		d	84	
Undecane	±	E		78	
Dodecane	+ +		d	68	
1-Dodecene	±			90	
Tridecane	+ + +			12	
Tetradecane	+ + + +	EE	dd	15	
1-Tetradecene	+ + +	E	dd	58	
Pentadecane	+ + + +			26	
Hexadecane	+ + +	EE	dd	52	m.p. 18°
1-Hexadecene	+ +	E		74	
Octadecane	+ + + +	EE	dd	40	m.p. 32°
1-Octadecene	+ + +		d	62	m.p. 16°
(B) 1-Bromododecane	+ +			65	(Lauryl bromide)
1-Iodotetradecane	+ + +			49	
1-Chlorotetradecane	+ +			58	
1-Bromohexadecane	+ + + +			31	(Cetyl bromide)
Dodecanethiol	+	E		77	(Lauryl mercaptan)
(C) 1,2-Epoxydodecane	0			105	
1-Dodecanol¶	0	N		78	m.p. 24–27°
1-Hexadecanol¶	0			82	m.p. 49° (cetyl alcohol)
3-Oxohexadecane**	+			85	
1-Octadecanol¶	+	All animals died		83	m.p. 57°
1,2-Epoxyoctadecane¶	+ + +			45	m.p. 30–32°
9-Octadecen-1-ol	0	N		97	Residual injury (foot) (oleyl alcohol)
'Ethoxylated oleyl¶ alcohol	0			85	m.p. 28–33°
Oleic acid	±	N		87	C_{18} mono-unsaturated fatty acid

* N = that of normal rats (no adjuvant); E = elevated by 50–250 per cent; EE = grossly elevated ($>4\times$N).

† See footnote to Table 1.

‡ DMA = drug metabolizing activity, measured by production of cyclophosphamide/aminopyrine metabolites after day 14. d = depressed by 25–50 per cent; dd = depressed by more than 50 per cent; n = 100 ± 25 per cent of concurrent control (normal animal without adjuvant).

§ Over days 0–14, with reference to weight-matched control rats of the same strain, not receiving adjuvant (see Table 1).

¶ Liquefied with 0·2 ml of hexane per g.

** Liquefied with 1 ml of hexane per g.

incidence in the front paws (after 3 weeks). The response to this severely arthritogenic (standard) adjuvant was remarkably consistent at different seasons of the year. However, the response to some minimally arthritogenic adjuvants did appear to vary somewhat over

a 24-month period. Thus an adjuvant constituted with *M. tuberculosis* in squalene or olive oil was least arthritogenic during two Spring seasons. Different strains of animals showed different rates of weight gain with and without an adjuvant inoculation (Table 1). The overall severity of disease was greatest in the Lewis rat and least in the Fischer rat. For routine purposes, most of the experimental 'adjuvants' were screened using the two outbred Wistar strains (CFN, HLW). Adjuvants yielding dubious results (arthritis) were then examined further using Lewis rats.

The average reduction in normal weight gain for groups of four or more rats proved to be the most consistent parameter (of those readily determined) for comparing the arthritogenicity of over a hundred oily materials and their derivatives (Tables 2–6). Detailed analyses of several other parameters (Table 7) supported this conclusion.

Statements concerning (co-)arthritogenicity refer to the properties of the named material when admixed with *M. tuberculosis* (or some other arthritogenic bacterial species). The most powerful co-arthritogens, e.g. mineral oil, 1-octadecene, pristane etc. did not induce arthritis, in the absence of mycobacteria.

(CO-)ARTHRITOGENICITY OF ACYCLIC HYDROCARBONS

Normal (i.e. unbranched) alkanes with twelve or more C atoms were arthritogenic. The arthritogenic threshold appeared to be ten carbon atoms (decane), though undecane ($C_{11}H_{24}$) proved marginally arthritogenic. The C_{12}–C_{16} alkenes (olefines) consistently induced less severe adjuvant disease than the corresponding saturated hydrocarbons (Table 2A). 1-Octadecene induced such a consistently severe disease that it could not always be distinguished from octadecane (a low melting solid) using arthritis induction as a bioassay. Redistilled 1-octadecene (b.p. 349°) and hexadecane (b.p. 287°) proved to be excellent substitutes for mineral oil in constituting highly reproducible arthritogenic adjuvants. Since hexane 'adjuvants' failed to induce arthritis in over thirty rats drawn from four susceptible strains, hexane was used to liquefy/solubilize various solids and waxes (with m.p. 30–70°) to permit the assessment of their intrinsic adjuvanticity and co-arthritogenicity.

Introduction of a single halogen or mercapto group did not affect the arthritogenicity of C_{12}–C_{16} hydrocarbons (Table 2B). The presence of oxygen in ketone, alcohol, oxirane (epoxide) or carboxyl groups effectively reduced or abolished the arthritogenicity of C_{12}–C_{18} hydrocarbons (Table 2C).

(CO-)ARTHRITOGENICITY OF CYCLIC HYDROCARBONS

The critical nature of the length of the alkane chain was clearly demonstrated on comparing the arthritogenicity of selected phenylalkanes (Table 3A): at least nine linear carbon atoms were required to induce arthritis. The phenyl group was itself devoid of activity and benzene or toluene could be used alternatively to hexane as dispersing media for examining the adjuvant/arthritogenic activity of solid/waxy compounds.

Various C_{10} and C_{12} cycloalkanes and cycloalkene derivatives were not arthritogenic (Table 3B,C). Arthritogenicity developed on partially saturating a C_{15} triene (trimethylcyclododecatriene). This limited group of observations confirmed the loss of arthritogenic

activity on inserting either an oxygen function or a double bond (see above). Cyclization also reduced the arthritogenic potential; compare the activity of dodecane (Table 2A) with that of the first three compounds listed in Table 3C. However, muscone, a 16-carbon cyclic ketone of animal origin, was definitely arthritogenic.

TABLE 3

ARTHRITOGENIC ACTIVITY OF SOME CYCLIC HYDROCARBONS AND THEIR DERIVATIVES, IN CONJUNCTION WITH *M. tuberculosis*

	Compound	Severity of arthritis†	Sleep time (days 13–17)*	DMA‡	Percentage average weight gain§	Comment (alternative name)
(A)	Benzene	0	N		88	
	Chlorbenzene	0			87	
	Toluene	0			85	
	1-Phenyl octane	0	N		78	
	1-Phenyl nonane	±	N		72	
	1-Phenyl decane	+ +	E		45	
	1-Phenyl dodecane	+ + +	EE		30	
(B)	1-Methylnaphthalene	0			84	
	Decahydronaphthalene	0	N		105	Saturated bicyclic C_{10} compound
	Cadinene	0	N		88	Unsaturated bicyclic C_{15} compound
(C)	*n*-Hexyl cyclohexane	±		n	85	
	Dicyclohexyl, $C_{12}H_{22}$	±		d	80	
	Cyclododecane (CDD)¶	0			100	Saturated monocyclic C_{12} compound
	1,2-Epoxy CDD	±			90	
	1-Cyclododecene (ΔCDD)	±			90	Iodine number = 150
	1,2-Epoxy-5,9-cyclododecadiene	0			97	
	1,5,9-Trimethylcyclododecatriene, $C_{15}H_{24}$	0			90	Iodine number = 373
	1,5,9-TrimethylΔCDD, $C_{15}H_{28}$	+ +			50	Iodine number = 123, b.p. = 222°
(D)	Cedrene, $C_{15}H_{24}$	+			80	Tricyclic monoene
	DL-Muscone, $C_{16}H_{30}O$	+			58	(3-Methyl-1-cyclopentadecanone)

* N = that of normal rats (no adjuvant); E = elevated by 50–250 per cent; EE = grossly elevated (> 4 × N).

† See footnote to Table 1.

‡ DMA = drug metabolizing activity, measured by production of cyclophosphamide/aminopyrine metabolites after day 14. d = depressed by 25–50 per cent; dd = depressed by more than 50 per cent; n = 100 ± 25 per cent of concurrent control (normal animals without adjuvant).

§ Over days 0–14, with reference to weight-matched control rats of the same strain, not receiving adjuvant (see Table 1).

¶ Liquefied with 0·2 ml of hexane per g.

(CO-)ARTHRITOGENICITY OF BRANCHED HYDROCARBONS AND SOME RELATED NATURAL PRODUCTS (POLYISOPRENOIDS)

The presence of double bonds and a hydroxyl group probably explains the inactivity of the di- and sesqui-terpenoid alcohols, geraniol and farnesol (Table 4A). 'Disparlure' (*cis*-7,8-epoxy-2-methyloctadecane), the sex attractant of the gypsy moth *Porthetria dispar* (Bierl, Beroza and Collier, 1970), was clearly arthritogenic. So were two other animal hydrocarbons (zoo-alkanes), squalene and pristane, which are both abundant in liver oils and

in skin secretions—squalene in human sebum, pristane in wool wax. Squalane, i.e. perhydrosqualene, was the most arthritogenic oil (with defined chemical composition) disclosed by these studies. The importance of double bonds and an alcohol group in lessening arthritogenicity is clearly shown on comparing squalene with squalane and phytol with pristane. Perbromosqualene, though saturated, was not arthritogenic.

TABLE 4

ARTHRITOGENIC ACTIVITY OF SOME BRANCHED HYDROCARBONS AND RELATED NATURAL PRODUCTS

	Materials	Severity of arthritis†	Sleep time (days 13–17)*	DMA‡	Percentage average weight gain§	Comment (alternative name)
(A)	Geraniol	0		n	105	$C_{10}H_{18}O$, a diene alcohol
	Farnesol (95 per cent)	±			82	$C_{15}H_{26}O$, a triene alcohol
	'Disparlure'	+ +			42	Slow development arthritis
	Squalene, $C_{30}H_{50}$ (purified)	+ + +	EE	dd	15	From thiourea adduct, iodine number = 380
	Squalene (commercial)	+ +	EE		55	Contained peroxides, iodine number = 400
	Perbromosqualene	0	N		96	Iodine number = 0·0
	Squalane, $C_{30}H_{62}$	+ + + +	EE	dd	5	(Perhydrosqualene)
	'Squalene oxides'	0			98	Mother liquors from squalene purification
	Pristane (norphytane)	+ + + +			40	(2,6,10,14-Tetramethylpentadecane)
	Phytol	0			95	(3,7,11,15-Tetramethyl-2-hexadecene-1-ol)
(B)	Petroleum jelly	+ +			57	('Vaseline')
	Wax (petroleum)¶	+ +			60	m.p. 47–53° ('Multiwax', Witco)
	Turpentine	+ +	EE		47	
	Turpentine substitute	0		n	90	Three commercial paint thinners
(C)	Vitamin K_1	+ + +			48	(Phytyl menadione)
	DL-α-Tocopherol	+ + +			52	(Vitamin E)
	Tocopherol acetate	+ + +			24	
	Retinol acetate**	+ +			40+	m.p. 57° (vitamin A_1 ester)
	Retinol palmitate	0			100	m.p. 28° (vitamin A_1 ester)

* N = that of normal rats (no adjuvant); E = elevated by 50–250 per cent; EE = grossly elevated ($>4\times$N).

† See footnote to Table 1.

‡ DMA = drug metabolizing activity, measured by production of cyclophosphamide/aminopyrine metabolites after day 14. d = depressed by 25–50 per cent; dd = depressed by more than 50 per cent; n = 100 ± 25 per cent of concurrent control (normal animals without adjuvant).

§ Over days 0–14, with reference to weight-matched control rats of the same strain, not receiving adjuvant (see Table 1).

¶ Liquefied with 0·2 ml of hexane per g.

** Liquefied with 1 ml of hexane per g.

Some waxy petroleum products proved arthritogenic. So did various 'oils of turpentine' derived from wood (Table 4B) but not the petroleum-derived substitutes.

Three fat-soluble vitamins (A, E and K) possessed intrinsic arthritogenic activity (Table 4C). Retinol palmitate, the preponderant vitamin A ester in fish liver oils, was apparently devoid of activity. The acetate ester, often used in synthetic vitamin supplements, was however arthritogenic.

(CO-)ARTHRITOGENICITY OF SOME OILS AND TRIGLYCERIDES

Croton oil and some aromatic oils (other than turpentine) proved to be bland as co-arthritogens, though they induced severe acute irritation (Table 5A). A liquid silicone of unknown composition, sold as a lock lubricant, was moderately potent. No correlation was found between the relative arthritogenicity and the unsaturated fatty acid content (indicated by the iodine number) of a number of plant fats (Table 5C). The iodine numbers

TABLE 5

ARTHRITOGENIC ACTIVITY OF SOME OILS AND TRIGLYCERIDES

	Material	Severity of arthritis†	Sleep time* (days 13–17)	DMA‡	Percentage average weight gain§	Comment (alternative name)
(A)	Croton oil	0			98	Residual foot swelling at injection site††
	Clove oil	0			100	
	Orange (rind) oil	0			78	Normal albumin levels (day 14)
	Lemon (rind) oil	0			80	
	Grapefruit (rind) oil	0			70	
	Cedarwood oil	0	N		92	
	Oil of origanum	±	N		88	
(B)	'Silicone oil'	++			22	A commercial lubricating oil
(C)	Coconut oil	+	E		73	Iodine number = 6 m.p. 28–29°
	Castor oil	±			93	Iodine number = 84
	Olive oil	+++	EE	dd	45	Iodine number = 85
	Arachis oil	++			65	Iodine number = 94
	Chaulmoogra oil	+			78	Iodine number = 98
	Sesame seed oil	++			67	Iodine number = 106
	Cottonseed oil	+			90	Iodine number = 115
	Corn oil	+++			40	Iodine number = 120
	Wheat germ oil	+		n	90	Iodine number = 132
	Safflower oil	+++		d	25	Iodine number = 139
	Cod liver oil	+			88	Iodine number = 155
	Oleomargarine‡‡	++	EE		55	Iodine number = 80, m.p. 32–34°
(D)	Tributyrin	0	N		80	
	Trioctanoin	0			85	
	Tridecanoin¶	++++			28	m.p. 28°
	Trilaurin§§	+++	E		50	m.p. 46°
	Trimyristin¶¶	+			75	m.p. 56°
	Tristearin¶¶	±			80	Hexahydro-triolein, m.p. 55°
	Triolein	++		dd	23	Iodine number = 91

* N = that of normal rats (no adjuvant); E = elevated by 50–250 per cent; EE = grossly elevated (>4 × N).

† See footnote to Table 1.

‡ DMA = drug metabolizing activity, measured by production of cyclophosphamide/aminopyrine metabolites after day 14. d = depressed by 25–50 per cent; dd = depressed by more than 50 per cent; n = 100 ± 25 per cent of concurrent control (normal animals without adjuvant).

§ Over days 0–14, with reference to weight-matched control rats of the same strain, not receiving adjuvant (see Table 1).

¶ Liquefied with 0·2 ml of hexane per g.

** Liquefied with 1 ml of hexane per g.

†† Croton oil only (no *M. tuberculosis*) did not cause any residual swelling (day 17) at the sites of injection.

‡‡ Safeway brand, contains hydrogenated soybean and cottonseed oils.

§§ Solubilized with 1 ml of benzene per g.

¶¶ Solubilized with 2 ml of benzene per g. These adjuvants were probably 'undervalued' owing to relatively low lipid content.

determined for each of these plant oils were all within the ranges recorded in the Merck Index.

Triolein (glycerol trioleate), a principal constitutent of olive oils and cocoa butter, did not seem to be as arthritogenic as whole olive oil (which also contains squalene and tocopherols). Some saturated triglycerides were also potent arthritogens (Table 5D).

TABLE 6

ARTHRITOGENIC ACTIVITY OF SOME CHOLESTEROL (CL) AND FATTY ACID ESTERS (EXCLUDING TRIGLYCERIDES) AND OTHER ANIMAL LIPIDS

	Material	Severity of arthritis†	Sleep time (days 13–17)*	DMA‡	Percentage average weight gain§	Comment (alternative name)
(A)	Methyl octanoate	0			100	Saturated C_8 ester
	Methyl laurate	0	N		100	Saturated C_{12} ester
	Methyl myristate	0			86	Saturated C_{14} ester
	Methyl pentadecanoate	±			64	Slow development arthritis
	Methyl palmitate	+			50	Saturated C_{16} ester, m.p. 30°
	Methyl stearate¶	++			65	Saturated C_{18} ester, m.p. 39°
	Methyl oleate	[0]	E	n	73	Monounsaturated C_{18} ester‡‡
	Methyl ricinoleate	0			95	(Methyl 12-hydroxyoleate)
	Methyl linoleate	0			64	Diunsaturated C_{18} ester
(B)	Butyl laurate	0	N		92	
	Butyl oleate	+++	EE	d	52	Iodine number = 81
	Butyl dibromoleate	±	E		84	Iodine number = 1·5.
	Butyl myristate	+			52	Residual foot swelling
	Butyl ricinoleate	0			95	
	Butyl palmitate	++++			29	
	Butyl stearate	++++			42	
	Lauryl laurate	+++	EE		58	
(C)	[Cholesterol (CL)§§	±			82]	
	CL 2-ethylhexylcarbonate	+++			72	
	CL 2-ethylhexanoate¶	++			62	m.p. 28–40°
	CL oleylcarbonate¶	++++	EE	dd	30	Arthritis at day 12
	CL oleate¶	+++	EE	dd	40	m.p. 17–46°
	CL myristate§§	+			67	m.p. 70–122°
	CL palmitate§§	+++			42	m.p. 77–146°
	CL linoleate¶	0	N	dd	90	m.p. 35–43°
	CL linolenate¶	0	N	n	90	m.p. 29–47°
(D)	Beeswax	++			64	m.p. 62°
	Lanolin	++,0,±			65,90,90	3 samples, 2 inactive
	Rat tail wax	+	E		80	m.p. 47°
	Rat intestinal lipids††	++++	EE		40	m.p. 20–22°
	Rat lymph node lipids††	+++			44	
	Rat adipose lipids††	+++			40	

* N = that of normal rats (no adjuvant); E = elevated by 50–250 per cent; EE = grossly elevated ($>4\times$N).

† See footnote to Table 1.

‡ DMA = drug metabolizing activity, measured by production of cyclophosphamide/aminopyrine metabolites after day 14. d = depressed by 25–50 per cent; dd = depressed by more than 50 per cent; n = 100 ± 25 per cent of concurrent control (normal animals without adjuvant).

§ Over days 0–14, with reference to weight-matched control rats of the same strain, not receiving adjuvant (see Table 1).

¶ Liquefied with 0·2 ml of hexane per g.

** Liquefied with 1 ml of hexane per g.

†† Solubilized in approximately 2 ml of benzene per g. of residue, obtained after extraction of tissue with benzene in a blendor and removal of the solvent from the benzene extracts.

‡‡ In extended testing of methyl oleate adjuvants, a mild arthritis developed in 30 per cent of Lewis rats and 20 per cent of the Wistar rats.

§§ Solubilized in approximately 2 ml of benzene per g.

(CO-)ARTHRITOGENIC ACTIVITY OF SOME ESTERS

Some esters which are not glycerides were also arthritogenic. The prime requirement was a fatty acid greater than C_{12}. *n*-Butyl esters were certainly more effective adjuvants than methyl esters. Again, desaturation and/or the presence of a hydroxyl group reduced arthritogenicity (compare oleates with stearates, Table 6A and B, and ricinoleate with oleate, Table 6B), but adding bromine to the double bond further reduced arthritogenicity (compare Tables 6B and 4A). Some low melting cholesterol esters which are also mesomorphic (or cholesteric) liquid crystals showed definite arthritogenic activity. Among these was cholesterol oleate, a major component of the ester fraction in human sebum (Nicolaides, Ansari and Rice, 1972). Neither cholesterol itself nor a non-ester (nematic) liquid crystal compound (*N*-paramethoxybenzylidene-parabutylaniline, m.p. 18–40°) was arthritogenic. Some animal epidermal waxes and the mixture of lipids extracted with benzene from washed rat intestines, hypertrophied lymph nodes and epididymal fat pads had arthritogenic activity (Table 6D, also see below). Lipids extracted into chloroform from these three latter tissues were far less arthritogenic than the benzene-extractable components.

PROPERTIES OF SOME COMPOUNDS IN THE ENVIRONMENT

A number of waxes (for floors, furniture and cars), cosmetic creams and skin lotions were examined for their arthritogenic potential. Significant arthritis (++) developed after inoculating *M. tuberculosis* with the following materials: a skin cream containing lanolin (Shepard Labs, Chicago), a skin lotion (Vaseline brand, Chesebrough Ponds, Incorporated, New York), Tiger Balm® ointment (m.p. 37–40°, Haw Por Brothers, Taiwan), materials containing turtle wax (e.g. car bumper wax). Several polishes containing acrylics and some containing silicones had no significant effect on the rats (weight gain 90 per cent controls). Hydrous lanolin preparations (skin moisturizers) seemed to be less arthritogenic than relatively anhydrous preparations but moisture content alone did not correlate with (lack of) arthritogenicity in the limited number of samples (five) examined. One of the most potent arthritogenic materials disclosed by this study was Johnson's Baby Oil® (Johnson and Johnson, New Brunswick, New Jersey; iodine number = 2·7), i.e. a mineral oil with lanolin, which fully matched the potency of four known paraffin oils (Squibb, Protol, Mazeol, Drakeol).

FURTHER ANALYSES OF SOME 'ADJUVANTS' FOR THEIR ABILITY TO INDUCE ADJUVANT DISEASE

There are good grounds for objecting to a structure–action relationship based only upon subjectively scoring the severity of arthritis and more objectively assessing disease status from failure to gain weight (Tables 2–6). Several other 'hallmarks' of the adjuvant disease were therefore quantified.

It was not possible to carry out routine biochemical and pathological analyses on the more than 800 rats used to compile the data concerning over 100 'adjuvants' listed in these previous tables. However approximately twenty of these adjuvants were subjected to much more intensive investigation and some (only) of the findings are summarized in Table 7. Some of the compounds named in this table were also used to prepare other

TABLE 7

FURTHER CHARACTERIZATION OF SOME ADJUVANTS BY THEIR ABILITY TO INITIATE SYSTEMIC DISEASE AND ARTHRITIS IN RATS AND MICE

Adjuvant = bacteria dispersed in:	Serum albumin*	Plasma inflammation units (±SDM)	Spleen weight*	Thymus weight (g)†	PPD skin test‡	Percentage average weight gains§ (Tables 1–6)	Arthritogenicity in rats with: *N. asteroides*	Arthritogenicity in rats with: BCG walls	Chronicity in mice¶
Saline	0·95±0·05	20	1·0	0·32±0·04	0	>80	0	0	0
Mineral oil	0·24±0·16	516±131	1·5	0·16±0·07	++	<30	++++	+	+, A, S
Hexane	0·95±0·14	75±91	1·15	0·28±0·03	±	100	0	0	0
Hexadecane	0·64±0·10	302±240	1·94	0·18±0·03	++	52	+++	+	±
Squalene, $C_{30}H_{50}$	0·85±0·15	260±70	1·2	0·29±0·03	++	15	++	N.D.	N.D.
Squalane, $C_{30}H_{62}$	0·51±0·14	430±40	1·8	0·26±0·08	+++	5	+++	N.D.	N.D.
Trimethyl ΔCDD**	0·50±0·03	350±130	1·6	0·23±0·04	+	50	N.D.	N.D.	N.D.
Trimethyl CDD triene**	0·85±0·05	20±5	1·1	0·35±0·02	0	90	N.D.	N.D.	N.D.
Methyl oleate	0·90±0·22	22±33	N.D.	N.D.	±	73	0	0	0
Butyl oleate	N.D.††	N.D.	N.D.	N.D.	+	52	N.D.	+	+
Glycerol trioleate	0·45±0·20	271±217	N.D.	N.D.	+	23	++	N.D.	N.D.
Cholesterol oleate	0·39±0·10	293±200	N.D.	N.D.	+	40	++	N.D.	N.D.
Pristane	0·40±0·05	400±70	1·7	0·18±0·06	+++	40	N.D.	N.D.	N.D.
Phytol	0·90±0·07	60±20	1·1	0·30±0·04	?	95	N.D.	N.D.	N.D.

The data (±SEM) was determined from at least four, and usually six animals per group.

The first six columns record data from rats inoculated with adjuvants containing *M. tuberculosis* 14–17 days previously.

* Values of normal littermates (no adj.) = 1·0; normal albumin levels were 37±3, 38±4, 41±9 mg/ml, respectively in Lewis, CFN and HLW rats; normal spleens averaged 1·5, 2·4 and 2·7 mg/gm body weight respectively in Lewis, CFN and HLW rats.

† Of 200–250 g animals.

‡ 50 μg PPD (Weybridge) in 0·1 ml saline injected subdermally 5–7 days after inoculating the adjuvant. Scored after 24 and 48 hours.

§ Over days 0–14, with reference to weight-matched control rats of the same strain, not receiving adjuvant (see Table 1).

¶ Sustained foot swelling when injected therein. A indicates distal arthritis (foot swelling) occasionally observed. S = also splenomegaly.

** Liquefied with 1 ml of hexane per g.

†† N.D. = not determined.

TABLE 8

EFFECT OF SOME 'ADJUVANTS' AND SOME OILS IN CAUSING EAE IN LEWIS RATS

With *M. tuberculosis*			Without *M. tuberculosis*			
Comment	Average weight gain (g) (day 0–12)*	Average score disease†	Oil	Average weight gain (g) (day 0–12)*	Average score disease†	Comment
M. tuberculosis in saline	+23	0	None	+37±7	0	GPSC only, n = 12
High mortality	−38	5·2	Mineral oil	−20	3·4	Mortality rare
	−45	4·0	Johnson's Baby Oil®	−8	3·0	
	+29	0	Hexane	+30	0	
	+23	1·0	Cyclohexane	+33	0	
	−32	1·4	Dodecane	0	0·6	
Considerable mortality	−72	5·5	Hexadecane	−45	4·2	
	N.D.		Phenyl octane	+38	0	
	N.D.		Phenyl decane	+25	0·7	
	N.D.		Phenyl dodecane	+18	1·6	
High mortality	−53	4·5	Squalene, $C_{30}H_{50}$	−33	4·5	Higher mortality than squalane
	N.D.		Perbromosqualene	+31	0	
	−61	5·1	Squalane, $C_{30}H_{62}$	−22	3·0	
High mortality	−39	4·4	Pristane	−31	5·5	High mortality
	+18	0·8	Phytol	+21	0·6	
	N.D.		Methyl stearate	+6	1·8	
	−50	4·0	Methyl oleate	−11	2·3	
	+12	1·7	Triolein	+22	< 1·0	
	−41	4·0	Butyl stearate	−4	2·5	
	−42	4·7	Butyl oleate	+11	1·0	
	−14	2·7	Cholesteryl oleate	−17	3·3	
	N.D.		Vitamin A acetate	+43	0	
	N.D.		Vitamin A palmitate	+44	0	
	−34	4·0	Vitamin K_1	+11	1·3	
	−49	4·1	Tocopherol acetate	+17	1·5	

Materials were inoculated with and without *M. tuberculosis*, as emulsions with guinea-pig spinal cord (GPSC), into inguinal lymph nodes.
Data was determined from groups of four or more animals.
* Of survivors at day 12.
† See Materials and Methods section. Maximum score = 6·0 (100% lethality).

'adjuvants' constituted with either purified BCG cell walls or whole *Nocardia asteroides* or *Corynebacteria rubrum*, which were then examined for their arthritogenic properties in rats. The adjuvants containing BCG walls were also injected into the right paws of five or more mice from several inbred and outbred lines* to assess their ability to induce a chronic foot swelling. In some of the mice there also developed a migratory polyarthritis, resembling a transient adjuvant disease (as normally observed in rats) particularly affecting the other rear paw with an incidence of 20–40 per cent.

By all criteria investigated, those arthritogenic adjuvants that were not constituted with mineral oil (MO) mimicked the MO adjuvants in causing lasting depression of hepatic drug metabolism (Beck and Whitehouse, 1973), chronic arthritis, profound hypoalbuminaemia, elevated 'inflammation units' and hyperfibrinogenaemia, splenomegaly with perisplenitis, thymic involution, adrenal hypertrophy, general lymphadenopathy (not documented in Table 7) and gross loss of abdominal fat. The arthritogenic adjuvants listed in Table 7 also initiated polyarthritis regardless of whether they were administered into the tail, inguinal lymph nodes, or the foot. By contrast, the marginal adjuvants generally exhibited a greater arthritogenic potential when given into the foot than when injected near the base of the tail.

The adjuvant disease initiated with both *M. tuberculosis* in hexadecane and with BCG cell walls dispersed in either mineral oil or hexadecane was successfully transferred in Lewis rats, 9 days after the adjuvant inoculation, using lymph node cells (harvested from the cervical, axillary, inguinal, mesenteric, lumbar and popliteal nodes) or thoracic duct lymphocytes, as previously described for adjuvant disease initiated with *M. tuberculosis* in mineral oil (Pearson and Wood, 1964; Whitehouse, Whitehouse and Pearson, 1969). These experiments will be reported in detail elsewhere.

Animals previously inoculated with one of several non-arthritogenic 'adjuvants' (constituted with *M. tuberculosis* and octane, epoxydodecane, phytol, decene, epoxycyclododecane, methyl oleate, cholesterol linolenate, turpentine substitutes) and shown to be PPD-negative, became PPD-positive and developed arthritis after being challenged with mineral oil or hexadecane adjuvants 21 days later. These particular non-arthritogenic adjuvants were apparently also non-tolerogenic, in contrast to some mineral oil adjuvants which may protect animals from developing an arthritis after a subsequent adjuvant challenge (Cozine, Stanfield, Stephens and Mazur, 1972, 1973).

ADJUVANTICITY FOR INDUCING EAE

Several representative adjuvants were emulsified with guinea-pig spinal cord. The potency of this combination for inducing allergic encephalomyelitis in Lewis rats was assessed by Newbould's (1965) technique of injecting the emulsion directly into the inguinal lymph nodes. The loss in weight of the animals over the next 12 days provided a useful quantifiable parameter of the severity of disease but it was significantly biased 'upwards'. Severely encephalitogenic emulsions, prepared with powerful adjuvants, often induced considerable mortality 2 or 3 days before day 12 and weight changes could only be averaged using the (less diseased) survivors. These figures given in the first column of

* The mice strains were A/J, BALB/J, CBA/J, C57Bl/6J, DBA/1J all from Jackson Laboratories, Bar Harbor, Maine; Swiss Webster from Hilltop Labs, Chatsworth, California; CD from Charles River Labs, Wilmington, Massachusetts.

data (Table 8), therefore represent minimal weight losses. Attempts to present data comparing a number of adjuvant–spinal cord preparations by the weight changes induced over a shorter period were considered unsatisfactory since (a) the rate of weight loss frequently differed at different times of year, even using a standard adjuvant and standard spinal cord preparation (freshly mixed just before use), and (b) certain adjuvants induced rather different rates of weight loss (i.e. particularly non-linear over days 6–12).

These adjuvant–spinal cord emulsions contained only 0·5 mg *M. tuberculosis*/ml and not more than 20–30 μl was injected per animal. This proved insufficient to induce signs of arthritis but, using mineral oil adjuvants, induced EAE in 100 per cent of the animals.

In the course of running controls, emulsions prepared with mineral oil and spinal cord alone (i.e. no *Mycobacteria*) were found to induce a less lethal, but nonetheless severe, form of EAE in normal Lewis rats. This milder 'bugless' disease could likewise be quantified by the weight loss over days 0–12, with much less bias since the mortality rates were much lower than when 'whole' adjuvants were used. Table 8 therefore records two sets of data obtained with (a) adjuvants (i.e. with *M. tuberculosis* in the encephalitogenic emulsion) and (b) with the oily vehicle alone.

Levine and Wenk (1965) reported EAE induction in pertussis-pretreated rats using an 'incomplete' adjuvant (containing emulsifier). Patterson and coworkers (Patterson, Drobish, Hanson and Jacobs, 1970) observed 25 per cent incidence of paralysis in Lewis rats injected with guinea-pig spinal cord and 'incomplete adjuvant' over the back and neck. As Table 8 indicates, emulsions of encephalitogen with mineral oil prepared without bacteria and applied directly to the lymphatic system via an intranodal injection gave a very reproducible disease in Lewis rats with $\geqslant$90 per cent incidence—from which the animals generally recovered by the 18th–21st day.

The threshold for effective adjuvanticity in rats among the normal alkanes appeared to be at C_{12} (dodecane); loosely agreeing with quite independent observations of the threshold for arthritogenicity in rats ($\geqslant C_{10}$ Table 2A) and the requirement of $>C_{13}$ alkane for inducing severe EAE in guinea-pigs (Shaw, Alvord and Kies, 1964a). Among the thirty adjuvants examined in this manner, there was in general rather good agreement between the potency of the adjuvant as an arthritogen *per se* and its efficacy as an (encephalitogenic) adjuvant for inducing EAE. Notable exceptions to this generalization included the following.

(1) Five samples of squalene which differed considerably in their rancidity (peroxide content) and arthritogenicity (see Table 4A) but showed remarkably similar adjuvant activity.

(2) The surprising potency of methyl oleate, even though it was a distinctly marginal co-arthritogen in concurrent assays (see Table 6A).

(3) The low potency of triolein as an encephalitogenic adjuvant when compared with both methyl and butyl oleates (and their respective arthritogenic potentials, Tables 5D and 6A,B).

Using the onset of 'bugless' disease as a criterion for intrinsic adjuvanticity, vitamin A esters appeared to be bland in this respect but vitamin E and K preparations showed some activity. Pristane, squalane and hexadecane were all very good substitutes for mineral oil both as conventional 'complete' adjuvants (with *Mycobacteria*) or as 'bugless' adjuvants. To refer to them as 'incomplete' adjuvants in this context, when used without an adjuvant micro-organism, would seem to be entirely inappropriate—for they were certainly sufficient in themselves, of initiating EAE in rats.

DISCUSSION

There are surprisingly few published reports indicating effective substitutes for mineral oil in preparing arthritogenic adjuvants. The failure of an unspecified 'pflanzenöl' (Keital, Rudolph, Kröning, Kretschmar and Jambor, 1969), possible efficacy of tocopherol (Glenn and Gray, 1965) and usefulness of olive oil (H. L. Murray, Evansville, private communication) support our own observations. The failure of other workers to note co-arthritogenic activity in other plant oils may perhaps be due either to the use of less arthritogenic bacterial components than the *M. tuberculosis* (human) strains used here, or to sampling oils which differed appreciably in composition from those we used, even though obtained from the same plant source (e.g. the oleate content of arachis oil may vary from 40–70 per cent total fatty acids, according to origin). The triglyceride structure of a fat, as well as its fatty acid composition, determines its atherogenic potential when fed to rabbits with cholesterol (Kritchevsky, Tepper, Vesselinovitch and Wissler, 1973); the same might be true for its arthritogenic and adjuvant potential.

Rats appear to be much more sensitive than guinea-pigs to the effect of incomplete adjuvants, i.e. mineral oil with emulsifier, either for inducing cell-mediated immunity (Patterson *et al.*, 1970) or depressing it (Asherson and Allwood, 1971). They are also the only small laboratory animal species to 'over-react' to complete adjuvants by developing adjuvant disease (Pearson, 1972) and the dysfunction of hepatic drug metabolism (Beck and Whitehouse, 1973). What significance should be attached to this peculiar sensitivity of the rat is at present unclear. Nevertheless the methods utilized in this study provide, in theory, excellent bioassays for monitoring oils and other readily solubilized lipids for their potential health hazard (to rats at least), either with or without a bacterial adjuvant principle.

CONCERNING THE 'ADJUVANTS'

All the adjuvants (i.e. dispersions of *M. tuberculosis* in an oily vehicle) discussed in this communication caused both an acute inflammation at the site of inoculation and caused the hexobarbital sleeping time of the animals to be significantly extended (by 150 per cent or more) within 48 hours. The hexobarbital sleep time reflects the activity of the liver microsomal drug-metabolizing enzymes; extended sleep times reflecting impaired drug metabolism. Thus both acute local and acute distal phenomena are elicited by mycobacterial 'adjuvants'—which certainly much exceeded the marginal effects of *Mycobacteria* alone (when given in saline). However only those adjuvants, designated 'arthritogenic', caused an enduring depression of hepatic drug metabolism (i.e. hexobarbital sleep times elevated for at least 2 weeks) and elicited some definite signs of allergic polyarthritis. The distinction to be drawn between the two classes of adjuvants (according to their arthritogenicity or otherwise), is therefore not so much one based on the initial severity, as one based on the ultimate duration, of the response(s) they elicit. The defence reactions provoked within the first few days by both types of adjuvants are indeed powerful ones, e.g. generating much local oedema, triggering several liver responses (reduction in albumin synthesis and drug metabolism, production of 'acute phase reactants' and elevated fibrinogen levels, etc.). However, the bland 'adjuvants' do not prevent a fairly rapid homeostatic restorative response at the injured site with concomitant normalization of albumin and fibrinogen levels, hexobarbital sleep times, etc. On the other hand, the

arthritogenic adjuvants (a) trigger an early PPD response, (b) retard local inflammation from subsiding, (c) retard normal growth, (d) generate a 'pathophoric' population of circulating lymphocytes able to transfer the disease, and (e) finally cause to be manifest a periarthritis, vasculitis, perisplenitis and ankylosis of the joints and anaemia (in this temporal sequence). Their chronic stressor activity is reflected in the thymic involution, adrenal hyperplasia and gross diminution of the body fat depots which they first provoke (and then sustain). In one sense, the adjuvant-intoxicated animal is an underresponder in failing to eradicate the mycobacterial irritant/stressor.

This study has shown remarkably little difference between the responses made by susceptible rat strains to each of forty or more arthritogenic adjuvants. There were remarkably few lipids among those examined which consistently yielded exceptional adjuvants, either causing an arthritis with little weight loss or the converse, or which otherwise failed generally to duplicate the Freund's type mineral oil adjuvants in their overall pathogenic characteristics. These findings suggest that the lipid component of the 'adjuvant' primarily modulates (rather than determines *de novo*) the intrinsic activity of the arthritogenic peptidoglycans (residing in the bacterial wall) which are essential for arthritis development.

Further experiments indicated that when an oily vehicle was a good co-arthritogen in conjunction with *M. tuberculosis*, it was also (i) a good co-arthritogen in conjunction with other bacterial arthritogens, and (ii) a good adjuvant (either by itself or in combination with *M. tuberculosis*) for inducing at least one other form of cell-mediated immunity, namely EAE, in the same strain of animals.

DETERMINANTS OF ARTHRITOGENICITY (ADJUVANTICITY)

Features of the *M. tuberculosis* cell wall and wax D which determine their arthritogenicity are discussed elsewhere (Koga and Pearson, 1973; Koga, Pearson, Ishibashi and Tanaka, 1974). This discussion will be restricted to considering the lipid milieu in which the mycobacterial arthritogen must be presented, if it is to be pathogenic. The presence of certain bland solvents such as benzene or hexane, even in considerable molecular excess, did not seem to prevent the arthritogenicity/adjuvanticity of many lipids being manifest. These solvents did not depress appreciably the arthritogenicity of many oils that were tested in combination with them. However, we cannot be sure that they did not perhaps moderate or enhance the intrinsic activity of those solid materials, which could only be administered to the rat as solutions/dispersions in benzene or hexane. With these reservations, certain conclusions can be drawn concerning the molecular determinants of arthritogenicity/adjuvanticity.

(1) Solid materials can be demonstrated to be co-arthritogens, even though there is disagreement about whether solid hydrocarbons can function as adjuvants (Freund, 1947; Shaw *et al.*, 1964a).

(2) Plant terpenoids, especially those that are polycyclic, multibranched, unsaturated and bear oxygen residues, are generally bland and innocuous for constituting adjuvants.

(3) Other lipids, especially those of animal origin, may be potentially more dangerous/autoprotective, especially those rich in saturated hydrocarbon moieties.

(4) Susceptibility to metabolism may explain why unsaturated or oxygenated lipids are weaker adjuvants than their saturated/desoxy analogues. However there remains to be explained the fact that many natural esters (Calam, 1971) and triglycerides are potentially

effective adjuvants, even though they are susceptible to degradation by ubiquitous lipases/esterases.

Some speculations

These data prompt some conjectures.

(1) The low melting waxes in sebum together with other sebaceous constituents (e.g. cholesterol oleate, squalene) may constitute an auto-adjuvant system, should the integument be broken. These components of sebum, if resorbed through the wound along with any adventitious micro-organisms, could perhaps boost the immune response to the penetrating micro-organisms.

(2) The increasing burdening of the environment with mineral greases, hydrocarbon sprays (e.g. containing insecticides), synthetic pheromones or insect lures including esters such as Hexalure (Keller, Sheets, Green and Jacobson, 1969) or hydrocarbons such as Disparlure or 9-tricosene, Muscalure (Carlson, Mayer, Silhack, James, Beroza and Bierl, 1971) or juvenile hormone analogues (Slade and Wilkinson, 1973), may be a mixed blessing.

(3) The wisdom of using (a) mineral oil products as skin and intestinal lubricants, (b) suppositories rich in cocoa butter (containing triolein, etc.) and even (c) oily parenteral drug formulations, e.g. aurothioglucose in sesame oil (Solganal) might be questioned.

(4) Likewise, the extensive ingestion of (a) synthetic fat-soluble vitamins or fish liver oils (also containing squalene, pristane) as nutritional supplements and (b) other indigestible lipids, may be of dubious value.

The presence of abnormal quantities of lipid on the body surfaces may not only trigger 'healthful' immunity but also 'harmful' autoimmunity in the presence of ubiquitous *Mycobacteria*, *Corynebacteria* and many of the common Gram-positive and Gram-negative organisms (e.g. present in the gastro-intestinal tract), which have been shown to (i) constitute effective adjuvants (for cell-mediated immunity) in combination with mineral oil (Shaw, Alvord, Fahlberg and Kies, 1964b) or (ii) have arthritogenic cell walls (Koga, Pearson, Narita and Kotani, 1973).

Other workers have clearly shown the adjuvanticity of vitamin A derivatives (administered in mineral oil) for initiating humoral responses in mice, with the palmitate ester being much less active than the alcohol (Dresser, 1968; Spitenagel and Allison, 1971). EAE studies suggest they have little or no adjuvanticity for cell-mediated immunity (used without *Mycobacteria*) in agreement with White's report (1972).

ADDENDUM

Hexadecane has been identified as the predominant straight chain alkane in two lots of a Pennsylvania mineral oil (Drakeol) which has been often used for preparing adjuvants, constituting 11–12 per cent by weight of the refined oil (O'Neill, Yamauchi, Cohen and Hardegree, 1972).

A reviewer raised the question of whether the EAE induced without *Mycobacteria* was truly a cell-mediated disease and not humorally induced. Lymph node cells harvested 8 days after inoculating encephalitogen/squalane emulsions into donor animals passively transferred EAE to syngeneic virgin rats. For this purpose the squalane had been freshly redistilled (b.p. 3490) and could be considered free from bacterial contamination. By this single criterion of being able to transfer the disease with washed lymphoid cells, an EAE

induced without *Mycobacteria* would seem to be equally cell-mediated as the EAE induced with mycobacterial adjuvants.

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